Small molecule inhibitor of *Vibrio cholerae* virulence and intestinal colonization

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Vibrio cholerae, the etiological agent of cholera, exhibits coordinate genetic regulation of several virulence determinants, most significantly the potent cholera toxin (CT), and the essential intestinal colonization factor, the toxin coregulated pilus (TCP). The regulatory pathways that influence expression of virulence genes in *V. cholerae* have been subject to detailed research in recent years, including the identification of ToxT as a key transcriptional activator of CT and TCP. A high throughput screen was developed based on a *ctx-tetA* reporter construction to identify small molecule inhibitors of ToxT within the 50,000 compound library of Chembridge. Two rounds of analysis led to 109 (0.22%) compounds which inhibited the growth of the *ctx-tetA* construction in the presence of tetracycline. Of these 109 compounds, 15 were selected based on their structure, effect on bacterial growth and inhibition of CT production. The lead compound, named Virstatin, {4-[N-(1,8-naphthalimide)]-n-butyric acid} had no effect on the growth of classical biotype strain O395 (600uM) or El Tor biotype C6706 (1200uM). The inhibition of CT and TcpA expression was studied under *in vitro* growth conditions that stimulate toxin and pilus expression. Minimum inhibitory concentrations of 3uM (for strain O395) and 40uM (for C6706) were determined to reduce toxin production to undetectable levels.

Activity of Virstatin

Virstatin was determined to inhibit ToxT by several methods including demonstration that virstatin inhibits ctx transcription, but not toxT transcription. Additionally, virstatin inhibited the activity of ToxT expressed under a heterologous promoter (pBADtoxT complementation of O395 *toxT*), and also prevented ToxT activation of ctx-lacZ cloned in *E. coli.*

An escape mutation of ToxT was identified by screening a mutated pBAD24-*toxT* library, allowing identification of a L113P mutation within the N-terminal domain of ToxT which resulted in relative resistance to virstatin. In comparison to the wild-type ToxT which was inhibited by 90% at 10ug/ml, the escape mutant (C6706mut) was unaffected by virstatin at 50ug/ml, and showed 80% activity at 60ug/ml.

The effect of virstatin was evaluated using the fully virulent El Tor cholera strain C6706 in the infant mice colonization model. Virstatin (50ug) was delivered with cholera inoculation with or without a small concentrated boost at 3.5-4 hours, comparing strain C6706wt to S533, a non-O1, non-O139 CT-negative strain that lacks the TCP island, and hence is both negative for *tcpA* and *toxT*. Experiments using virstatin at 500ug/ml (which is not toxic *in vitro*), showed that drug applied at inoculation and boosted at 3.5-4 h reduced recovery of bacteria by 4-logs, an effect that did not occur with control escape mutant $C6706_{mut}$.

Finally, virstatin decreased C6706 colonization in infant mice by 3 logs at 36 and 60 hours evern when administered 12h after inoculation of the mice with bacteria, suggesting that in established infection, virstatin could have utility in clearing infection even after disease is diagnosed.

Activity of alrestatin

We have synthesized and tested 30 structural analogues of virstatin and have identified an analogue {4-[N-(1,8-naphthalimide)]-n-ethanoic acid}, alrestatin, which demonstrates similar potency as virstatin in vitro, with an MIC of 4uM, for inhibition of ToxT. In the infant mouse model of infection, alrestatin was similarly active both in early and late inoculation, though higher doses were required to obtain the same in vivo efficacy, requiring 100ug/mL dosing in the infant mouse. When extrapolated to a 60 kg person, this would translate to 1 gm po tid of alrestatin compared to 500 mg po tid for virstatin.

Alrestatin was developed by Ayerst as an aldose reductause inhibitor for diabetic neuropathy. It was administered to diabetics at 1-1000 mg/kg/day, with an LD50 of 2500 mg/kg in rats. In addition, diabetics were treated up to 4 months, with the major toxicity being phototoxicity. It is no longer in development due to short serum half-life. However, we are currently pursuing the possibility of obtaining GMP material from Ayerst for testing in *V. cholerae* challenge studies.

In conclusion, a high-throughput phenotypic screen can be used to identify small molecule virulence inhibitors that exhibit *in vivo* efficacy against bacterial infection in the absence of any chemical or structural target information. A new class of highly targeted, small molecule inhibitors of virulence may provide novel approaches to circumvent traditional routes to antimicrobial resistance, and offer a therapeutic option that minimizes the perturbation of intestinal flora. Treatment of infections with antivirulence drugs may limit the risk of treatment failure, and will not engender widespread resistance to conventional antibiotics. Small molecules that inhibit virulence gene expression may synergize with traditional drugs to help clear infection. Members of this new class of drugs can be defined as *'A synthetic or natural compound that inhibits the expression of microbial virulence factors in vivo.'*